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DEMONSTRATION OF (Na^++K^+)-SENSITIVE ATPase ACTIVITY IN RABBIT POLYMORPHONUCLEAR LEUKOCYTE MEMBRANES

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Summary

Contrary to earlier reports, we have been able to demonstrate the presence of (Na^++K^+)-activated, ouabain-inhibited ATPase activity in rabbit polymorphonuclear leukocyte membranes. These results coupled with others suggest that (Na^++K^+)-ATPase and not cation-sensitive phosphatases are responsible for the regulation of the electrolyte content in these cells.

It is generally agreed that in mammalian cells the intracellular concentration of K^+ is much higher than the corresponding value in the extracellular fluid whereas the situation for Na^+ is reversed. These concentration gradients are maintained by the "Na,K pump" which is driven by metabolic energy derived from the hydrolysis of ATP by membrane-associated (Mg^{2+}+Na^++K^+)-sensitive adenosine triphosphatases ((Na^++K^+)-sensitive, ouabain-inhibited ATPase). In spite of the fact that rabbit polymorphonuclear leukocytes maintain similar concentration gradients of Na and K across their cell membranes, many attempts have failed in the past to demonstrate the presence of (Na^++K^+)-ATPase activity in the plasma membrane of these cells [1—3]. Furthermore, it was suggested that cation-sensitive phosphatases and not (Na^++K^+)-ATPase are responsible for the regulation of the electrolyte content in these cells [2]. These studies were undertaken to re-examine this apparent inconsistency. We have found, contrary to earlier reports that these membranes contain small components of (Na^++K^+)-activated, ouabain-inhibited ATPase activity.

Polymorphonuclear leukocytes were obtained from white albino rabbits (2—3 Kg) which were injected intraperitoneally with 300—500 ml of sterile isotonic saline solution containing glycogen (0.5 g/l). The peritoneal exudate was collected 16 h later in a heparinized flask. The leukocyte-rich exudate

was strained through four layers of cheesecloth to remove debris. The suspension was gently centrifuged at 500 × *g* for 2 min and then the supernatant was removed and replaced by equal volumes of isotonic buffered NH_4 Cl. The packed cells were resuspended with a Pasteur pipette and kept at room temperature for 5 min. This procedure was necessary to hemolyze red cells [4,5]. The suspension was centrifuged for 5 min at 270 × *g*, and the cells were immediately washed once with a buffered sucrose solution (0.25 M sucrose, 10 mM Tris·HCl, pH 7.4).

After the washing, the pellet was suspended in a small volume of the buffered-sucrose solution and transferred to a glass homogenizer tube. The polymorphonuclear leukocytes were pelleted in the homogenizer tube and the supernatant was discarded. The concentrated pellet was homogenized on ice with a teflon pestle at approximately 950 rev./min at 5 strokes at a time until the majority of the polymorphonuclear leukocytes were broken as determined by microscopic examination.

The homogenate was diluted to 12 ml with a solution of 0.25 M sucrose, 5 mM Tris·HCl, 1 mM EDTA and layered on a 30%—40%—57% (by weight) sucrose gradient. All of the sucrose solutions contained 5 mM Tris·HCl and 1 mM EDTA. The gradients were centrifuged at 105 000 × *g* for 1 h at 4°C. Band I was formed between the suspending medium (0.25 M sucrose) and 30% sucrose, band II between the 30% and 40% sucrose, and band III between the 40% and 57% sucrose. The material designated as band II is referred to as plasma membrane fraction and that designated as band III is cytoplasmic membrane fraction [2]. We have also verified this identification by measuring the plasma membrane enzyme activity 5′-nucleotidase. The bands were transferred to clean centrifuge tubes and diluted with cold 16.4 mM Tris·HCl. The bands were concentrated by centrifugation at 105 000 × *g* for 1.5 h. The pellets were then resuspended in 16.4 mM Tris solution. Protein concentration was determined by the method of Lowry et al. [6] using bovine serum albumin as standard.

$(Na^+ + K^+)$-sensitive ATPase activity was determined by measuring the amount of inorganic phosphate released from the enzymatic hydrolysis of ATP. The specific activity of the membranes were measured under the following conditions: (1) Mg^{2+}, Na^+; (2) Mg^{2+}, K^+; (3) Mg^{2+}, Na^+, K^+; (4) Mg^{2+}, Na^+, K^+, ouabain. Final ionic concentrations were: Mg^{2+} 0.26 mM, Na^+ 100 mM, K^+ 10 mM, ouabain 0.1 mM. All samples contained 10 mM Tris·HCl pH 8.6, and $5 \cdot 10^{-5}$ M EGTA. The final ATP concentration was 0.2 mM and the final volume was 0.5 ml. All samples, before and after incubation, were kept on ice.

The reaction was started by the addition of the membrane, with final protein concentrations ranging from 0.015 to 0.035 mg protein per ml. Incubation was at 37°C for 10 min. The reaction was stopped by the addition of 0.15 ml of 30% trichloroacetic acid (w/v). The samples were centrifuged at 15 000 × *g* for 10 min in a refrigerated Sorvall RC-2B centrifuge. The phosphate concentration of the supernatant was determined by the method of Ames, except that the color development was at 4°C for 15 h to minimize ATP hydrolysis [7].

The various components of the ATPase activity found in fraction II are

summarized in Table I. The results in this table represent ten different membrane samples. It is quite clear from the table that this fraction contains a $(Na^+ + K^+)$-activated, ouabain-inhibited component. It meets all the requirements (Na^+-activated, K^+-activated, ouabain-inhibited) of the commonly known $(Na^+ + K^+)$-ATPase which is involved in the maintenance of the Na^+ and K^+ concentration gradients across cell plasma membrane. Although, in these membranes, the activity of the $(Na^+ + K^+)$-ATPase component is only a small fraction of the total, the absolute value is at least two-fold higher than generally found in human red cell membrane [8]. This $(Na^+ + K^+)$-activated, ouabain-inhibited ATPase activity could not be due to possible contamination of the leukocyte preparation by red cell membranes. After the red cells were hemolyzed with NH_4Cl, the suspension was centrifuged for 5 min at 270 × *g*.

TABLE I

ATPase ACTIVITY IN FRACTION II OF RABBIT POLYMORPHONUCLEAR LEUKOCYTE MEMBRANES

The activity is present as μmol P_i per mg protein per hour. Concentrations are as follows: Mg^{2+} 0.26 mM, Na^+ 100 mM, K^+ 10 mM, ouabain 10^{-4} M. n.d., not determined. Ouabain-sensitivity refers to the activity which is inhibited by ouabain and respresents the difference between columns 5 and 4. Means are given with the standard error of the mean.

Expt.	$Mg^{2+} + Na^+$	$Mg^{2+} + K^+$	$Mg^{2+} + Na^+ + K^+$	$Mg^{2+} + Na^+ + K^+$ + ouabain	Ouabain-sensitivity
1	2.4	n.d.	3.3	2.3	1.0
2	4.5	n.d.	6.1	4.4	1.7
3	6.3	n.d.	7.1	6.7	0.4
4	3.7	4.0	5.2	3.2	2.0
5	2.7	3.0	3.5	2.8	0.7
6	—	5.6	6.5	5.9	0.6
7	5.2	n.d.	5.6	5.1	0.5
8	5.6	5.3	6.4	5.7	0.7
9	5.9	5.6	6.3	5.3	1.0
10	5.7	6.1	6.9	6.2	0.7
Mean	4.8	4.9	5.7	4.7	0.93
S.E.M.	0.48	0.48	0.42	0.48	0.17
P^*	<0.001	<0.001		<0.001	

*Using paired sample test columns 2,3 and 5 are significantly different from 4.

At this speed and time of centrifugation, most if not all cell membranes or ghosts will stay in the supernatant. More important, based on the value of $(Na^+ + K^+)$-ATPase activity in mammalian red cell membranes and the degree of contamination of our leukocyte preparation, their maximum contribution to the value of $(Na^+ + K^+)$-activated, ouabain-inhibited ATPase activity in leukocyte membrane would be less than 5%.

The presence of $(Na^+ + K^+)$-ATPase activity in rabbit polymorphonuclear membrane is consistent with the transport properties of these membranes for Na^+ and K^+ (Naccache, P. and coworkers, unpublished data). We find (Naccache, P. and coworkers), that K^+ influx and Na^+ efflux are ouabain sensitive and Na^+ efflux is critically dependent on the level of extracellular K^+. These are the classical characteristics of the Na^+, K^+ pump commonly found in various biological plasma membranes.

The various components of the ATPase activity in fractions I and III were also studied. As evident from Table II, there is little if any $(Na^+ + K^+)$-

TABLE II

ATPase ACTIVITY IN FRACTIONS I, II AND III OF RABBIT POLYMORPHONUCLEAR LEUKOCYTE MEMBRANES

The activity is present as μmol P_i per mg protein per hour. Concentrations are as follows: Mg^{2+} 0.26 mM, Na^+ 100 mM, K^+ 10 mM, ouabain 10^{-4} M. Number in parenthesis refers to number of experiments. Means are given with the standard error of the mean.

Fraction No.	$Mg^{2+} + Na^+$	$Mg^{2+} + K^+$	$Mg^{2+} + Na^+ + K^+$	Mg^{2+} $Na^+ + K^+$ + ouabain
I	4.5 ± 0.40 (5)	4.7 ± 0.40 (5)	4.8 ± 0.40 (5)	4.6 ± 0.35 (5)
II	4.8 ± 0.48 (9)	4.9 ± 0.48 (6)	5.7 ± 0.42 (10)	4.7 ± 0.48 (10)
III	3.3 ± 0.61 (5)	1.4 ± 0.30 (4)	3.30 ± 0.30 (5)	3.0 ± 0.60 (5)

activated, ouabain-inhibited ATPase activity in either of these two fractions.

Based on these studies, we conclude that the observed Na^+, K^+ concentration gradients across rabbit leukocytes membranes are maintained by means of the "Na^+, K^+ pump" which is driven by a metabolic energy derived from the hydrolysis of ATP by membrane-associated (Na^++K^+)-activated, ouabain-inhibited ATPase.

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